Chapter 6

Conclusions, speculations and future perspectives

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The best way to have a good idea is to have lots of ideas.

Linus Pauling

In this thesis we describe novel features of hepatic peroxisomes that are relevant for normal liver function. Moreover, we describe changes in peroxisomal function in livers of patients with acute or chronic hepatitis that may provide cellular protection during the diseased condition. Here we discuss our most important findings and speculate on research directions that may further delineate the basic cell biological mechanisms involved, as well as the relevance for patients with liver disease.

In chapter 2 we show that both human and rat bile acid-CoA:amino acid N-acyltransferase (BAAT) are peroxisomal enzymes in the liver. The subcellular localization of BAAT/Baat has been studied by various experimental approaches and still a consensus has not been reached. Without doubt, a certain portion of BAAT resides in peroxisomes as it co-fractionates with peroxisomal markers in both biochemical and microscopical analyses (1, 2). However, the presence of a significant amount of BAAT in the cytosol is the controversial issue. The presence or absence of such a cytosolic pool is of critical importance for the intracellular transport of unconjugated bile salt that enter the hepatocyte from the portal blood. A sole peroxisomal location of BAAT would require import of these unconjugated bile salts into peroxisomes for conjugation to glycine or taurine. If significant amounts of BAAT resided in the cytosol, such extension of the enterohepatic transport of bile salts would not be required. Evidence for a cytosolic pool of BAAT comes from cell fractionation studies and heterologous expression of a GFP-BAAT fusion protein in human fibroblasts (3). Peroxisomes have been shown to be particular fragile organelles that are easily damaged during cell fractionation experiments (4, 5). BAAT present in fractions containing the cytosolic proteins may therefore originate from peroxisomes that got damaged. It is thus crucial that the subcellular location of BAAT is (also) determined by other techniques.

We chose to study this by immunofluorescence microscopy and digitonin-permeabilization experiments. Using these techniques we found that in normal human and rat hepatocytes BAAT/Baat is primarily, if not solely, located in peroxisomes. In addition, a GFP-BAAT fusion protein efficiently targets to peroxisomes when expressed in primary hepatocytes. Also in our hands, this fusion protein showed poor peroxisomal sorting efficiency in human fibroblast as described by others (6). This observation points out the importance of the cellular environment for the efficient subcellular sorting of (peroxisomal) proteins, which is often not considered in such studies. Fibroblasts are relatively easy to obtain from patients and can be cultured *in vitro*. They are widely used to study peroxisome function and biogenesis in patients with peroxisomal disorders. In light of our findings it is important to realize that organ- and/or cell-specific functions, such as bile salt conjugation studied here, may not be well represented by fibroblasts even for a conserved process like targeting of peroxisomal enzymes. At present we do not know the factors or conditions that make BAAT a peroxisomal protein in hepatocytes which are absent or limiting in fibroblasts. The C-terminal targeting signal, SQL, may be a contributing factor. The SQL signal present in BAAT is a degenerate form of the typical and strongest peroxisomal targeting signal, SKL. If the protein import machinery is rate-limiting and competition arises between newly synthesised proteins that contain peroxisomal targeting signals, BAAT may be at the losing end and accumulate in the cytosol. Such a condition may occur when components of the import machinery that physically interact with the targeting signal, such as the PTS1-receptor Pex5p, are limiting. A comprehensive comparison of Pex5p levels in various organs or cell types, for instance fibroblasts versus hepatocytes, has not been performed yet. Identification of such limiting factors or conditions is required to understand the cell-specific difference in the sorting efficiency of BAAT.

The peroxisome proliferative agent, clofibrate has been reported to change the distribution of BAAT between peroxisomes and the cytosol (7). Moreover, this effect appears to be different in male and female rats. Clofibrate treatment induced an accumulation of BAAT in peroxisomes in male rats whereas the opposite, BAAT accumulation in the cytosol, was observed in female rats. Again, these studies included only cell fractionation experiments. It is therefore needed that such studies are analyzed by other techniques to firmly establish the differential localisation of BAAT depending on gender and/or fibrate-treatment.

The ultimate proof that bile salts shuttle through the peroxisome for conjugation would be to show that externally added unconjugated bile acids would actually enter peroxisomes. This is currently being studied in our laboratory. Transport of conjugated bile salts through peroxisomal membranes has recently been demonstrated. This is an essential and final step in the *de novo* biosynthesis of bile salts that makes them available for enterohepatic cycling. Thus, experimental approaches become available to identify and characterize the transporters responsible for bile salt transport in and out of peroxisomes and to study their role in bile salt homeostasis.

In man, BAAT deficiency leads to high levels of serum unconjugated bile acids and increased urinary excretion of bile acids, composed chiefly of unconjugated and glucuronidated/sulfated bile acids. The patients present with pruritus, fat malabsorption and vitamin K coagulopathy (8, 9). Although the clinical symptoms of the mutations have been described, nothing is known about the cellular effects of impaired bile salt conjugation in hepatocytes. Do bile salt intermediates accumulate in hepatic peroxisomes of these patients and does this affect peroxisome function or integrity? Detailed cell biological studies on patient material will be required to answer these questions.

Apart form the question whether a cytosolic pool of BAAT exists, we do not know whether the subcellular location of the protein has any influence on BAAT activity. The cytosol and the peroxisomal matrix each provide a unique microenvironment that may affect BAAT function. In Zellweger patients, peroxisome biogenesis is disturbed and matrix proteins accumulate in the cytosol, including BAAT. As a consequence, bile acid biosynthesis is affected in these patients leading to accumulation of C_{27} bile acid intermediates. However, the reduced pool of primary bile salts (CA and CDCA) appears normally conjugated to glycine or taurine (10, 11), indicating that BAAT is not inactive in the cytosol. Still, BAAT activity may be (partly) compromised when residing in the cytosol. This is suggested by the observation that Pex2-/- knock-out mice, an animal model of Zellweger syndrome, accumulate unconjugated bile acids in serum, liver and bile when fed a diet containing cholic acid or ursodeoxycholic acid (12). It remains to be determined whether the peroxisome deficiency reduces the protein level of BAAT or rather that the activity of the protein is compromised in the cytosol.

The consequences of impaired transport of bile salts in and/or out the peroxisomes remain hypothetical. It may lead to accumulation of bile salt intermediates and therefore resemble bile salt synthesis defects (13). Bile salts may also accumulate to such high levels inside peroxisomes that they will damage the organelle leading to deficient peroxisome function and biogenesis. Such a phenotype would resemble peroxisome deficiency as observed in Zellweger syndrome but than only in the liver, more precisely in the hepatocytes. These hypothetical effects would typically lead to liver damage that may progress to chronic liver disease. Maybe some cases in this large and heterogeneous group of patients may be attributable to defects in transport of bile salts through peroxisomal membranes.

In chapter 3 we explored the possibility that one or more members of the Solute Carrier Family 10 (SLC10) is/are a novel bile salt transporter(s). In particular, we were interested to identify a bile salt transporter for one of the organelles, for instance in the peroxisomal membrane. The sodium-dependent taurocholate co-transporting polypeptide (NTCP) and the apical sodium-dependent bile salt transporter (ASBT) are the founding members of the SLC10 protein family. Analysis of public databases reveals that this family contains 4 uncharacterized hypothetical proteins, SLC10-A3 to -A6 that by sequence similarity are good candidates to be bile salt transporters. After determining the tissue expression profile, we focussed our attention to SLC10A5. It is expressed in all the digestive tract organs, with highest expression in the liver. The expression profile of SLC10A5/Slc10a5 in human, mouse and rat perfectly mirrored that of the bile salt sensor, the Farnesoid X Receptor (FXR). Moreover, human SLC10A5 appeared to be regulated by FXR. Unfortunately, antibodies raised against synthetic fragments form SLC10A5 did not detect a specific protein in Western blot experiments nor did we detect antibody-specific signals by immunofluorescence microscopy. GFP-tagged SLC10A5 did accumulate intracellularly when artificially expressed in HepG2 cells but did not clearly colocalize with a specific organelle. Though expression and regulation clearly point to a role in bile salt homeostasis, we have not been able to characterize its substrate transporting activity, nor its subcellular location. The intracellular transport of bile salts and the proteins involved therefore remains merely a black box.

Bile salt transport by the peroxisomal membrane has recently been demonstrated (14). The positive identification of the protein responsible for this activity now depends on further characterization of hypothetical bile salt transporters using this assay system or purification of the bile salt-transporting activity from peroxisomal membranes and identification of the protein by reverse genetics. At the moment it is unclear whether this protein will be a member of the known protein families that contain bile salt transporters, SLC10, ABC, OATP (SLCO), OATs (SLC22) or OST.

Our characterization of the SLC10 proteins also identified SLC10A4 as a protein that is particularly expressed in brain. This protein has also been identified and characterized by others, who also found the specific brain-expression of this protein (15). These authors expressed the protein in *Xenopus* oocytes and performed substrate transport assays. They tested taurocholate, but were unable to detect any significant transport of this bile salt. In fact, they did not report on any other substrate that may be transported by this protein. The brain-specific expression may be associated with the brain-specific activity of CYP46 that converts cholesterol into 24-hydroxy-cholesterol (16). This is an important activity that controls cholesterol homeostasis in the brain. 24-hydroxy-cholesterol is subsequently transported out of the brain to the circulation. It enters the liver where it is further metabolized in the bile salt biosynthesis pathway to the primary bile salts chenodeoxycholate or cholate. It would be therefore interesting to test whether SLC10A4 would be able to transport bile salt intermediates, in particular 24-hydroxy-cholesterol. Future experiments need to clarify this issue.

In chapter 4, we studied the possible association of peroxisomal membrane proteins (PMPs) with specific lipid microdomains, so-called lipid rafts. These lipid domains are enriched in cholesterol and are characterized by their unextractability by certain detergents (Triton X100 or Lubrol WX) at 4^{o}C and their buoyancy after flotation gradient centrifugation (17, 18). We analyzed 4 different PMPs: two substrate transporters of the ATP-binding Cassette (ABC) family (PMP70 and ALDP) (19) and two peroxins involved in peroxisome biogenesis that

physically interact (Pex13p and Pex14p) (20). We found that PMP70 and Pex14p showed the most significant association with detergent-resistant lipid rafts. The proteins seem to co-exist in these lipid rafts. ALDP was also associated with lipid rafts, but only after Lubrol extraction, not with Triton X-100. Pex13p did not show any association with lipid rafts. This is the first description of lipid rafts in mammalian peroxisomes. Peroxisomal lipid rafts have been described before for the yeast *Yarrowia lipolytica* (21). Those *Yl*-lipid rafts contain Pex1p, Pex2p and Pex6p and were proposed to be involved in peroxisome maturation processes requiring fusion of distinct peroxisome precursor vesicles. No differentiation in lipid raft-association was described for the *Y. lipolytica* PMPs. We show the two highly homologous proteins, PMP70 and ALDP, show different lipid rafts-association characteristics. PMP70 and ALDP are both so-called ABC half-transporters that are supposed to dimerize to make a functional transporter. Two additional peroxisomal ABC half-transporters are known, PMP70R (or PMP69) and ALDRP. It is controversial whether these peroxisomal ABC transporters are active as homodimers or heterodimers (22, 23). Since we show that the lipid environment is different for PMP70 and ALDP it is unlikely that they constitute functional transporters together. Depletion of cholesterol leads to dissociation of all PMPs from the lipid rafts. In addition, it led to missorting of ALDP, while PMP70 remained peroxisomal. Thus, lipid raft-association is important for targeting of, at least a subpopulation of, PMPs. Since this is the first description of lipid raft-association of PMPs in the human peroxisomal membrane it raises more questions than it answers. What lipids constitute peroxisomal lipid rafts and what is the difference between PMP70/Pex14p- and ALDP-containing lipid rafts? How do the peroxisomal lipid rafts assemble and is there a putative role for the endoplasmic reticulum? What determines the association of PMPs to lipid rafts? Are lipid rafts important for peroxisomal function and are lipid raft assembly defects possibly involved in the development of peroxisome-associated diseases like Zellweger syndrome and adrenoleukodystrophy? To start with the last question, it seems inevitable that impaired lipid raft assembly in the peroxisomal membrane will result in peroxisome malfunctioning. ALDP sorting is strongly effected in cholesterol-depleted cells in which lipid raft-association of all tested PMPs is disrupted. Mutations in the ALD (*ABCD1*) gene cause X-linked adrenoleukodystrophy (24) and impaired sorting of the protein will undoubtedly have the same consequence. There is very little known about the process of lipid raft assembly. They are shown to spontaneously assemble in synthetic membranes (25), but it is conceivable that it is a regulated process *in vivo* with specific proteins involved. Lipid rafts have been shown to assemble in the ER and travel via the Golgi apparatus in secretory vesicles to the plasma membrane (26). Also in organelles involved in the endocytic pathway rafts have been identified (27). Since it is now generally accepted that peroxisomes may arise from the ER (28), it is tempting to speculate that the ER may play a role in assembly of peroxisomal lipid rafts. PMP70 has been detected in specialized domains of the ER in dendritic cells (29) and it is interesting to know whether it is already associated with lipid rafts at that subcellular location. A detailed analysis of the dynamics of the association to lipid rafts may further strengthen the functional link between the ER and peroxisomes. Taken together, this work described the existence of peroxisomal lipid rafts and opens a new field of research to determine their role in peroxisome function and biogenesis.

In chapter 5, we found that during inflammation of the liver, and in particular during acute hepatitis, catalase accumulates in the cytosol of hepatocytes. Catalase is normally a peroxisomal enzyme that converts H_2O_2 generated by peroxisomal oxidases into H_2O and O_2 (30, 31). Thus, redistribution of catalase to the cytosol is potentially a harmful situation because

peroxisomes have lost their maximum capacity to decompose H_2O_2. On the other hand, hepatitis is often associated with oxidative stress generated by neutrophils and infiltrating cells (32). Catalase is also generally regarded as an anti-oxidant enzyme protecting cells against this kind of extra-peroxisomal oxidative stress. Still, its peroxisomal location is often not taken into account. With our observation of subcellular redistribution of catalase during hepatitis we set out to determine whether the subcellular location of catalase actually matters with respect to protection against extracellular oxidative stress. Using HepG2 cells in *in vitro* experiments, we made two observations. Firstly, in normal cells, peroxisomal catalase does assist in protecting cells against exogenous added H_2O_2. This reconfirms the general anti-oxidant function of catalase also for disease-induced oxidative stress. Secondly, cells with cytosolic catalase show much more resistance against H_2O_2-induced toxicity compared to cells with equal amounts of catalase in peroxisomes. Linking this to the observation that catalase accumulates in the cytosol of hepatocytes during hepatitis, implies that this might serve as an adaptation of the cell to cope with conditions of increased oxidative stress. The key message of this study is that cells may use subcellular relocation of an anti-oxidant enzyme as a 3rd and novel adaptive mechanism, independently from regulating gene/protein expression or protein activity. Our observation does not stand alone. Catalase has also been shown to accumulate in the cytosol of aging fibroblasts. In fact, there are also indications that in the aging liver, catalase accumulates in the cytosol (33). This has been suggested to cause an oxidative imbalance and therefore may aid to the course of aging. A putative protective role of this adaptation has not been considered in these studies, but our present data indicate these cell may be actually better off. Catalase relocation would then be advantageous in conditions where the source of oxidative stress is extra-peroxisomal. For instance, the major source of oxidative stress during aging is thought to originate form mitochondria. In line with this, over-expression of catalase artificially targeted to mitochondria in transgenic mice significantly increases the animals' maximum life span, whereas overexpression of peroxisomal catalase does not (34).

As the benefit of cytosolic catalase in cell protection has been clearly proven and its role during hepatitis can be envisioned, many questions remain to be answered. What causes the cytosolic accumulation of catalase? Is catalase leaking out of peroxisomes or is the import of newly synthesized catalase blocked? Is this selective for catalase or do other peroxisomal enzymes undergo the same fate? Oxidative stress is such a common contributor to disease development and one may question whether catalase relocation is observed in other pathologies as well? We found that exposure to H_2O_2 *per se* does not lead to significant pools of catalase on short term. In fact, catalase remained peroxisomal in H_2O_2-treated necrotic cells. It will be useful to analyse the effect of other disease markers, like cytokines or supraphysiological bile salt concentrations, on the subcellular location of catalase and other peroxisomal enzymes. The mechanism that causes catalase to accumulate in the cytosol is not clear. The peroxisomal targeting signal of catalase is known to be relatively weak compared to the classic peroxisomal targeting signal type 1 (PTS1), C-terminal –SKL (35). Therefore, the binding to the PTS1 receptor Pex5p could be a determining factor. Decreased amounts of PEX5p may allow import of proteins with a strong PTS1 but limit the import of catalase. Obviously, it is now important to study regulation of components of the peroxisomal import machinery, including Pex5p, during hepatitis. An alternative mechanism would be that peroxisomal catalase would leak out of peroxisomes to the cytosol. This would suggest that hepatitis causes physical damage to the peroxisomal membrane. Within peroxisomes, catalase has been shown to reside closely to the peroxisomal membrane (36). Therefore, it could be one of the first markers of peroxisomal damage leaking to the cytosol. In a different context, this is

actually also observed in cell fractionation studies where large amounts of catalase end up in the fractions containing cytosolic proteins (37). In this respect, catalase can be regarded as one of the "leakiest" peroxisomal enzymes. Whatever mechanism gives rise to cytosolic catalase, peroxisomes have a unique way of re-importing proteins from the cytosol. Peroxisomal proteins can be imported in a completely folded state (17). In fact, PTS-less proteins can piggy-back on PTS-containing proteins into peroxisomes (17). Thus, active, tetrameric catalase can be imported again allowing the re-establishment of peroxisomal functions together with adequate anti-oxidant capacity after the pathophysiological conditions have been cleared.

In conclusion of this thesis, peroxisomes in the liver have revealed some new features that inspired us to study this in detail. The severity of peroxisomal biogenesis disorders already underscores the key function that this organelle plays in normal liver physiology. Our data hint to additional roles. In addition to bile salt *de novo* biosynthesis, we show that peroxisomes are involved in recycling of bile salts reabsorbed from the intestine, and indicate the need of hitherto unidentified peroxisomal bile salt transporters. We also show the flexibility of the organelle in response to oxidative stress, suggesting relocation of catalase as a response mechanism, rather than a cause of oxidative imbalance. Finally, we demonstrate the association of peroxisomal proteins to lipid microdomains. While illuminating novel aspects of peroxisome function in the liver, our observations will require further study to determine their relevance for human (patho)physiology.

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